OPTIMIZATION OF NITROGEN FIXATION IN GENETICALLY ENGINEERED AZOTOBACTER VINELANDII THROUGH MEDIA COMPOSITION MODIFICATION

by

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A thesis submitted to Johns Hopkins University in conformity with the requirements for the degree of Master of Science in Engineering

Baltimore, Maryland

October 2019

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Abstract

Although nitrogen is a vital metabolite, its naturally occurring form, nitrogen gas, is unusable by organisms. Through the process of nitrogen fixation, diazotrophic organisms can reduce nitrogen gas into ammonia, which can then be utilized by other organisms. One such diazotroph, Azotobacter Vinelandii, shows potential to be further utilized as a renewable source of nitrogen in co-culture systems. Through the modification of media composition, we will attempt to optimize the excretion of ammonium by A. Vinelandii 163 and subsequently evaluate its potential as a renewable nitrogen source in a co-culture environment.

The ammonium production of Azotobacter Vinelandii is directly dependent on its nitrogen fixing enzyme, nitrogenase, which can be synthesized with three variant forms of dinitrogenase. In A. Vinelandii 163, the molybdenum nitrogenase expresses a nif+ phenotype, which allows for the continuous production of ammonium without the negative feedback inhibition of the NifL protein. After experimentation, we have determined that the presence of iron in our media was sufficient enough to promote the synthesis of iron nitrogenase and thus suppress the nif+ phenotype. In order to optimize the ammonium production of Azotobacter Vinelandii 163, the strain must be grown in media devoid of iron. Therefore, with its current condition, A. Vinelandii 163 cannot be utilized as a nitrogen fixing species in co-culture systems that require iron sources. If A. Vinelandii 163 is to be used as a nitrogen source in co-culture conditions, then the extent of iron's effect on the repressed synthesis of molybdenum nitrogenase would need to be explored.

Advisor: Dr. Michael Betenbaugh

Reader: Dr. Chao Wang

Acknowledgements

I would like to thank Dr. Michael Betenbaugh for being an amazing mentor and advisor. Throughout my time at Hopkins, he has always motivated me in my pursuit of knowledge and established the type of outstanding educator that I seek to become. I would also like to thank Dr. Chao Wang, who, on multiple occasions, has set my chaotic mind at ease with his words of encouragement. To the rest of the Betenbaugh Lab, you all have been wonderful friends and mentors. To Jack Jenkins, Liqun Jiang, Yifeng Hu, and the rest of Team Algae, I must thank you from the bottom of my heart for all the advice that you have given me throughout our time together. Finally, I must thank Evelyn Chiu, Kristyn Green, Zachary Rodriguez, Esther Rodriguez, and all my other friends and family who have supported me through thick and thin.

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Introduction

Nitrogen is a vital metabolite, utilized in two major macromolecules: proteins and nucleic acids. However, in its naturally occurring form, nitrogen gas is unusable by organisms, meaning that the metabolite must be reduced into an organic form, such as ammonia or nitrate, through the process of nitrogen fixation (Kim & Rees, 1994). This task can be accomplished through industrial means, but with significant demands of pressure and energy. Fortunately, diazotrophic microorganisms have found a niche function in nature as biological nitrogen fixators, which require minimal energy usage (Goodsell, 2002). One such diazotroph, Azotobacter Vinelandii, has the potential to be utilized further as a renewable source of nitrogen in co-culture systems.

The wild type Azotobacter Vinelandii DJ is a diazotrophic microorganism, capable of fixing atmospheric nitrogen. The nitrogen fixation process is accomplished via the nitrogenase enzyme, which exists in three variants, each with its own unique metalloprotein. The wild type strain, A. Vinelandii DJ, fixes enough nitrogen to satisfy the growth needs of the cell. Through genetic modification, researchers have engineered a nif+ strains of A. Vinelandii, AZBB163, which will continuously fix nitrogen and subsequently excrete ammonium (Bali, Blanco, Hill, & Kennedy, 1992). Through the modification of media composition, we will attempt to optimize the excretion of ammonium by A. Vinelandii 163 and subsequently evaluate its potential as a renewable nitrogen source in a co-culture environment.

Background

2.1 Nitrogen Fixation via the Nitrogenase Enzyme

In industry, the reduction of nitrogen gas to ammonia requires high temperatures of 300 to 500 °C and pressures of up to 300 atm (Goodsell, 2002). Through the utilization of the nitrogenase enzyme, the reduction reaction's energy consumption is reduced to the usage of two ATP molecules per electron over the course of a three step process. This energy reduction is achieved through the two metalloprotein structure of nitrogenase, which allows for a multistage reduction reaction as electrons are transferred from donor to enzyme and, finally, to the nitrogen molecule. These two metalloproteins are known as the dinitrogenase and the dinitrogenase reductase (DNR) (Bali, Blanco, Hill, & Kennedy, 1992).

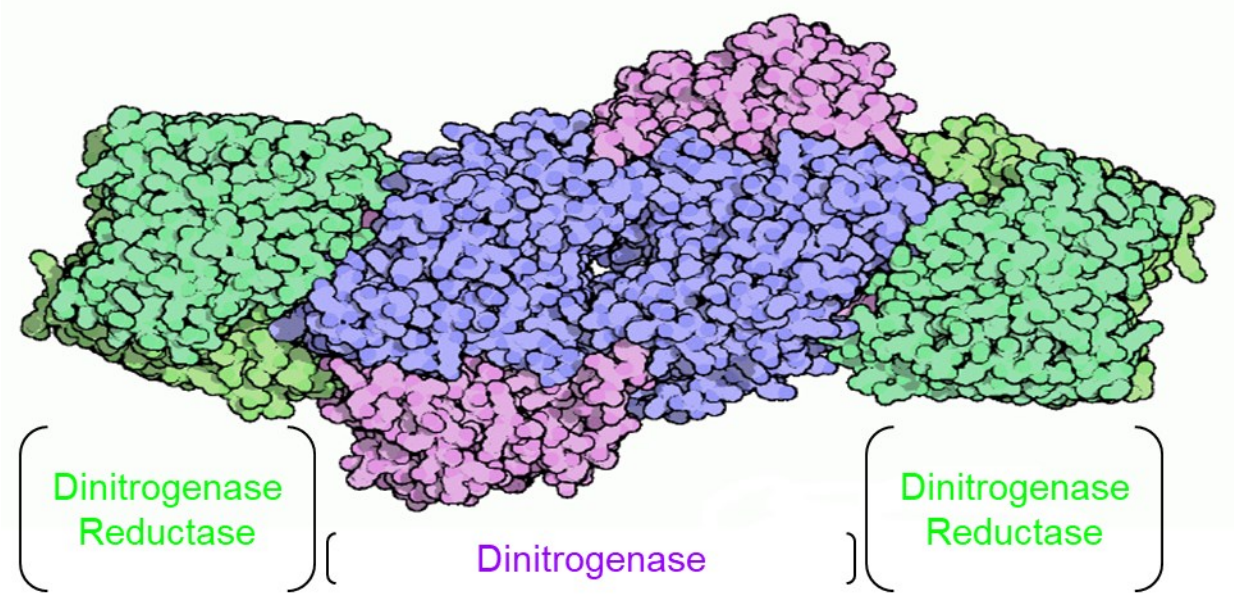

Figure 1: Structure of Nitrogenase Enzyme

The dinitrogenase reductase enzyme is responsible for acquiring energy for the nitrogen reduction and obtaining the electrons to achieve the reduction. Firstly, DNR receives two electrons from an electron donor. In vivo, this electron donor is usually a flavodoxin, a bacterial protein. DNR must then transfer two electrons from itself to the dinitrogenase metalloprotein

through an ATP dependent process in which, two ATP molecules are consumed for each electron transferred.

$$N_2 + 8H^+ + 8e^- + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

Equation 1: The reduction of Nitrogen gas to Ammonium via the Nitrogenase Enzyme

In conjunction with the primary reduction reaction, a side reaction occurs that allows for the formation of hydrogen gas through the reduction of protons. This reduction of protons will also lead to the consumption of more ATP molecules. Once electrons are transferred, dinitrogenase can thusly reduce the nitrogen molecule to its final form of ammonia (Noar & Bruno-Barcena, 2018). This is the standard nitrogen fixation reaction mechanism of nitrogenase. However, more can be said about the nitrogenase enzyme in A. Vinelandii as the dinitrogenase metalloprotein comes in three variations.

2.2 The Three Variants of Dinitrogenase in A. Vinelandii

In A. Vinelandii, dinitrogenase can be synthesized in three variations, each differing in their metal-iron cofactor: molybdenum, vanadium, or iron. The availability of these metals in the environment promote the expression and repression of the dinitrogenase that is synthesized. For example, when molybdenum is present in the media the synthesis of molybdenum dinitrogenase is favored over the other two. In this case, the synthesis of molybdenum dinitrogenase suppresses the synthesis of vanadium and iron dinitrogenase. If molybdenum is not available or is in low concentrations, the synthesis of vanadium nitrogenase will be favored over iron dinitrogenase (Kennedy & Dean, 1991).

Functionally, the three variants mentioned above reduce nitrogen gas to ammonium identically. There is little kinetic variation between them, as the rate limiting step in the nitrogen fixation process is during the initial dinitrogenase reductase reaction (Hageman & H, 1978). In

addition, the only difference between the three dinitrogenase physically is found in the metal iron cofactor which assists in the reduction of the substrate. In the molybdenum and vanadium dinitrogenase, molybdenum and vanadium can be found bound to the iron in the cofactor respectively. In the iron dinitrogenase, however, the cofactor only contains iron and a compound that acts homologous to molybdenum and vanadium (Hu & Ribbe, 2015).

2.3 Genetic Transcription of Nitrogenase in A. Vinelandii

Nitrogenase transcription is encoded by the nif, vnf, and anf family of genes for molybdenum, vanadium, and iron nitrogenase respectively (Kennedy & Dean, 1991). Each genetic family is homologous to the other two and for that purpose, the nif gene family of molybdenum nitrogenase will be discussed here to describe all three. The initial promotion of nitrogenase synthesis is dependent on the transcription nifLA operon, whose transcription is dependent on the phosphorylation of the NtRC protein. Once transcribed, the nifLA operon will produce the NifA and NifL proteins. The NifA is required for the active expression of the nifHDK genes, which are then directly responsible for the synthesis of dinitrogenase and dinitrogenase reductase (Hu & Ribbe, 2015). Once synthesized, the nitrogenase enzyme can then fix atmospheric nitrogen to ammonium. In wild type A. Vinelandii DJ, this process leads to the inhibitory feedback loop of the NifL protein.

There are two means of inhibiting the synthesis of nitrogenase enzyme. In the first case, the NifL protein can act as the inhibitory agent by preventing the activity of NifA. As the nitrogenase enzyme fixes nitrogen to satisfy its metabolic needs, it will subsequently build up ammonium within the cell. At ammonium concentrations of 5 μM or greater, the NifL protein will bind to the NifA protein and repress its activity. In the second case, which, occurs at ammonium concentration of 200 μM or greater, the NtRC protein becomes dephosphorylated,

preventing the transcription of the nifLA operon and thusly also preventing the expression of nifA gene and the subsequence synthesis of nitrogenase. Both of these negative feedback loops prevent the over production of ammonia and assure that nitrogenase will produce enough ammonia for its own metabolic needs (Bali, Blanco, Hill, & Kennedy, 1992).

Materials and Methods

3.1 Strain and Growth Conditions

Azotobacter Vinelandii 163 was provided by Dr. Brett Barney from the University of Minnesota. A. Vinelandii 163 is the third generational strain from the original wild type strain, A. Vinelandii DJ. The strain, AZBB163, spontaneously mutated to include a nif+ phenotype, which increased production and excretion of ammonium, and a kanamycin resistance gene. The nif+ phenotype prevents the negative feedback inhibition of the NifL protein and allows for the independent expression of nifA without NtrC. Thusly, this phenotype allows for the nitrogenase enzyme to not be regulated by the concentration of ammonium in the system (Barney, Eberhart, Ohlert, Knutson, & Plunkett, 2015).

To cultivate a pure stock of A. Vinelandii, AZBB163 was grown on Burk's medium plates with Kanamycin at 30-37 °C. In liquid cultures, AZBB163 was also grown in Burk's medium at a temperature of 30 °C, a pH of 7, and agitated with a magnetic stir bar at a rate of 150 rpm.

Two control medium were considered during this experiment, B media and BG-11 without a nitrogen source and with added sucrose. Under experimental conditions, AzBB163 was grown using modified versions of B media and BG-11-N. BG-11-N+Sucrose medium is prepared using following the recipe: 0.04 g/L K2HPO4·3H2O, 0.02g/L NaCO3, 0.075 g/L MgSO4·7H2O, 0.035g/L CaCl2, 0.00024 g/L citric acid, 0.0004 g/L EDTA·2H2O, 0.00024 g/L $FeCl_3$, 20 g/L sucrose, and 1mL trace metal solution per liter. The trace metal solution includes 2.86 g/L H3BO3, 1.81 g/L MnCl2·4H2O, 0.222 g/L ZnSO4·7H2O, 0.39 g/L NaMoO4·2H2O, 0.079 g/L CuSO4·5H2O, 49.4 mg/L and Co(NO3)2·6H2O (BG-11 Medium for Blue Gree Algae, n.d.). B medium is prepared following the recipe: 0.2 g/L KH_2PO_4, 0.8 g/L K_2HPO_4, 20

g/L sucrose, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.09 g/L $CaCl_2 \cdot 2H_2O$, 0.22 mg/L $Na_2MoO_4 \cdot H_2O$, and 5 mg/L $FeSO_4 \cdot 7H_2O$ (HIMEDIA, 2015). When measuring the effect of pH on growth, HEPES buffer is added by using a 300 g/L stock solution with pH 7.0, 8.0, or 8.9.

3.2 Measurement and analysis

3.2.1 Quantification of A. Vinelandii 163

A. Vinlandii growth in axenic culture is quantified by measuring the optical density at 600 nm with SpectraMax M3 Plate Reader. The cell density has the following correlation with OD_{600}:

$$Cells/mL = 2.71 \times 10^7 \times OD_{600}$$

Equation 2: Correlation between OD600 and Cell Density of A. Vinelandii 163

3.2.2 Ammonium Measurements

The free ammonium concentration is measured by combining 100 µL samples with 100 µL of assay reagent, which is prepared by combining 270 mg of dissolved phthalic dicarboxaldehyde in 5 mL of ethanol, 100 mL of 0.2 M phosphate buffer at pH 7.3, and 50 µL of Beta-mercaptoethanol (Corbin, 1984). Once the sample and assays are mixed, they are left to react at room temperature in darkness for 30 minutes. Molecular Devices SPECTRAmax GEMINIXPS fluorescence spectrophotometer is used to analyze the samples with 410 nm excitation wavelength and 472 nm emission wavelength. Standard curve is prepared by measuring solution with known concentration of NH_4Cl solutions.

Results

4.1 Comparison of Control Medias

To optimize the conditions that result in highest ammonium production, we first established the growth and ammonium excretion capabilities of A. Vinelandii 163 in two control medium, Burk's media and Bg-11-N with sucrose. We grew A. Vinelandii 163 in these control conditions for seven days, recording the optical densities and ammonium concentrations each day.

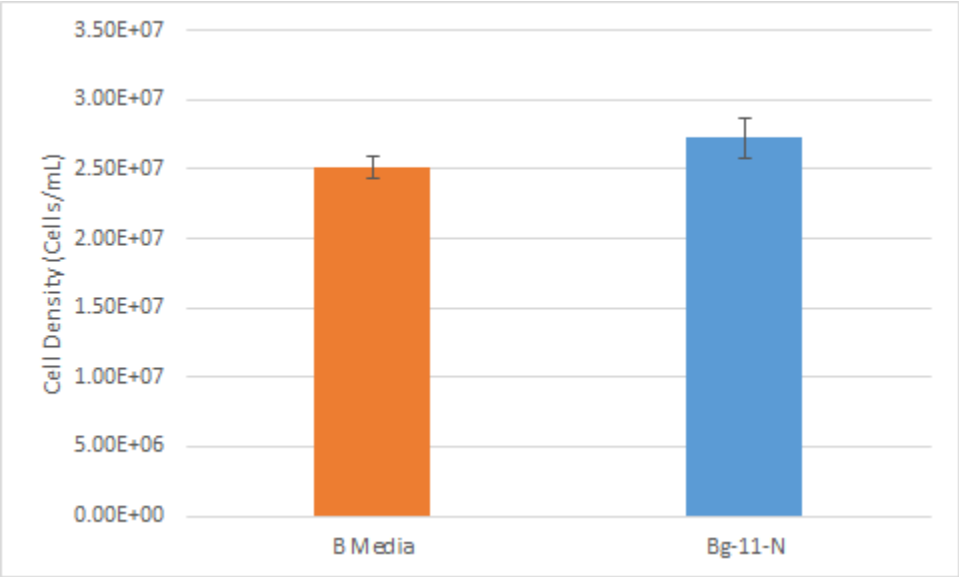

Figure 2: A. Vinelandii 163 growth under control media conditions

Cultures of A. Vinelandii 163 are grown at 28 °C, 150 rpm, in their respective media conditions, without antibiotics, and with 20 g/L sucrose for seven days. To ensure strain purity, cultures are taken from B media culture plates that contain kanamycin. The growth of A. Vinelandii is monitored by measuring the optical density at 600 nm. Displayed above is the cell density at day seven calculated using **Equation 2**.

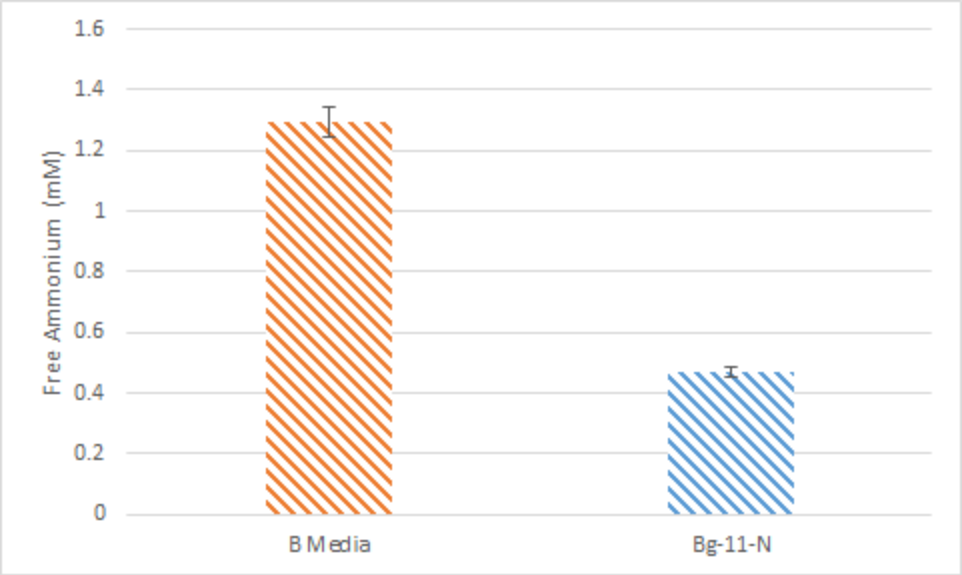

Figure 3: Effect of control media conditions on A. Vinelandii 163 ammonium production

Cultures of A. Vinelandii 163 are grown at 28 °C, 150 rpm, in their respective media conditions, without antibiotics, and with 20 g/L sucrose for 7 days. To ensure strain purity, cultures are taken from B media culture plates that contain kanamycin. Displayed above is the free ammonium concentration at day seven. The ammonium concentration is measured using the reagent assay containing 270 mg of phthalic dicarboxaldehyde, 5 mL of ethanol, 100 mL of 0.2 M phosphate buffer at 7.3 pH, and 50 µL of Beta-mercaptoethanol.

As shown in *figure 2*, the final cell densities at day seven are 2.51×10^7cells/mL and 2.72×10^7cells/mL for B media and Bg11-N respectively. Although, the cell density in the Bg11-N condition was higher, *figure 3* shows that A. Vinelandii 163 in B media produces a greater concentration of ammonium than Bg11-N by day seven at 1.29 mM and 0.466 mM, respectively.

4.2 Effect of pH buffer on Ammonium Production

To evaluate the effect that pH posed to the ammonium production of A. Vinelandii 163, we added HEPES buffer to B media at varying pH buffers: pH 7, pH 8, and pH 8.9.

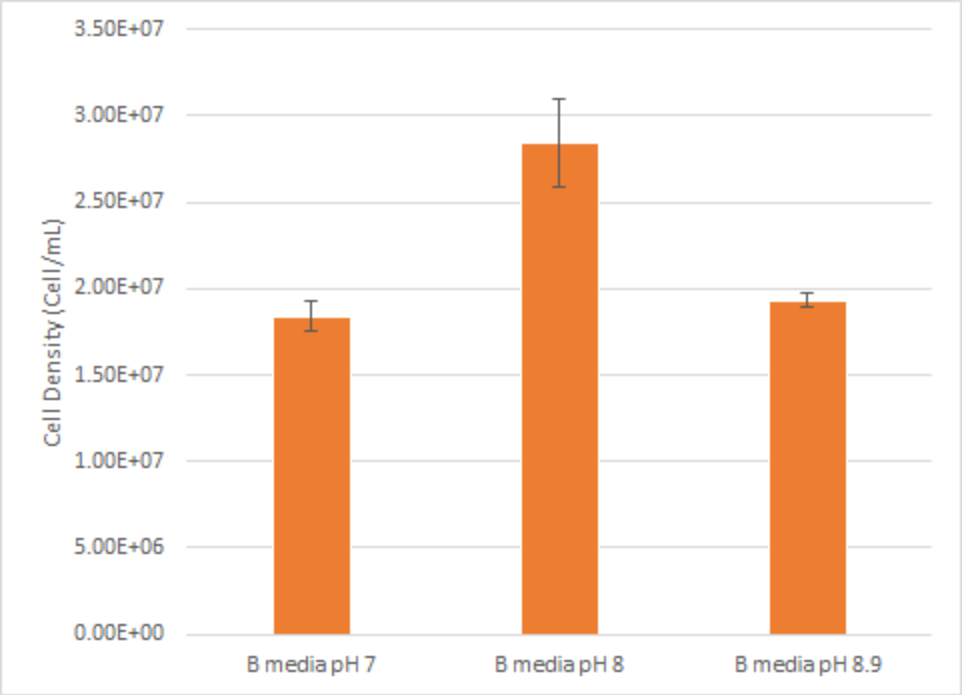

Figure 4: A. Vinelandii 163 growth under varying pH conditions

Cultures of A. Vinelandii 163 are grown at 28 °C, 150 rpm, in their respective media conditions, without antibiotics, and with 20 g/L sucrose for seven days. To ensure strain purity, cultures are taken from B media culture plates that contain kanamycin. The growth of A. Vinelandii is monitored by measuring the optical density at 600 nm. Displayed above is the cell density at day seven calculated using **Equation 2**.

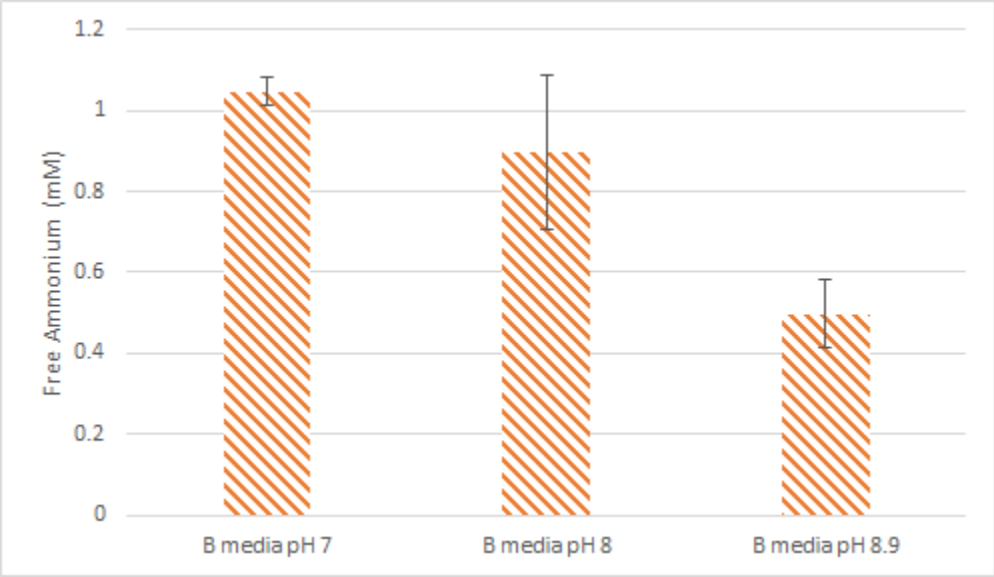

Figure 5: Effect of pH on A. Vinelandii 163 ammonium production

Cultures of A. Vinelandii 163 are grown at 28 °C, 150 rpm, in their respective media conditions, without antibiotics, and with 20 g/L sucrose for 7 days. To ensure strain purity, cultures are taken from B media culture plates that contain kanamycin. Displayed above is the free ammonium concentration at day seven. The ammonium concentration is measured using the reagent assay containing 270 mg of phthalic dicarboxaldehyde, 5 mL of ethanol, 100 mL of 0.2 M phosphate buffer at 7.3 pH, and 50 µL of Beta-mercaptoethanol.

As seen in *figure 5*, the ammonium production was at its highest at 1.04 mM when HEPES buffer pH 7 was added to B media. Free ammonium concentration then decreased as the pH of the media rises with the lowest concentration at 0.49 mM at pH 8.9. The pH of the media had more varied effects on the cell density of A. Vinelandii 163 with a pH 8 having the highest cell density at 2.72×10^7cells/mL.

4.3 Modification of Bg11-N Media composition

As Bg11-N produced less ammonium after seven days, we decided to modify the recipe for Bg11-N first to improve ammonium production to ranges similar to B media's ammonium production. This was accomplished by removing grouped categories of the media's composition, "Trace Metals" and "Stock 4" as detailed in *figure 6*. Additionally, we added metals from B media, $MgSO_4$, $FeSO_4$, and Na_2MoO_4.

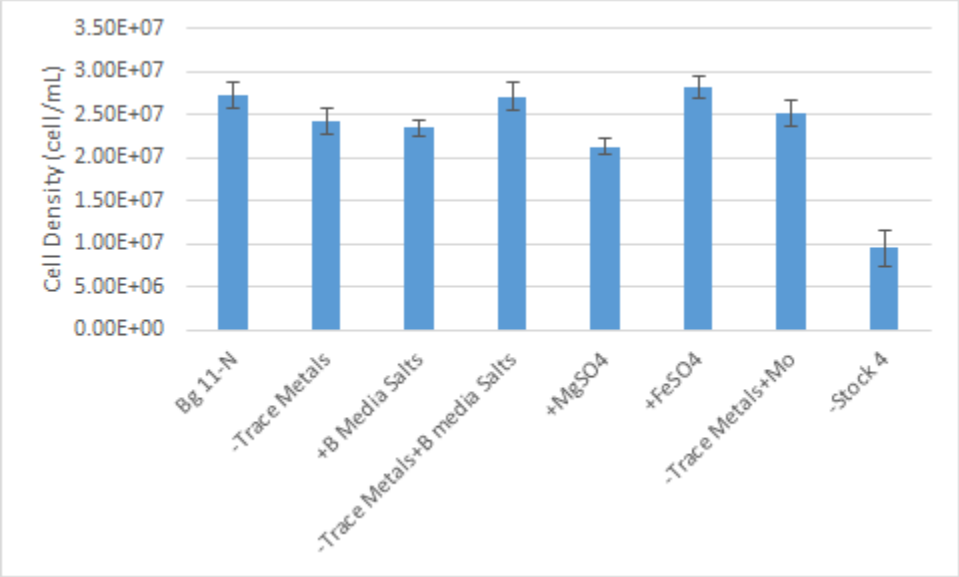

Figure 6: A. Vinelandii 163 growth under modified Bg11-N media composition

Cultures of A. Vinelandii 163 are grown at 28 °C, 150 rpm, in their respective Bg11-N media conditions, without antibiotics, and with 20 g/L sucrose for 7 days. To ensure strain purity, cultures are taken from B media culture plates that contain kanamycin. The growth of A. Vinelandii is monitored by measuring the optical density at 600 nm. Displayed above is the cell density at day seven calculated using **Equation 2**. "-Trace Metals" refers to the removal of H_3BO_3, $MnCl_2*4H_2O$, $ZnSO_4*7H_2O$, $Na_2MoO_4*2H_2O$, $CuSO_4*5H_2O$, and $Co(NO_3)_2*6H_2O$ from Bg11-N media. "+B Media Salts" refers to the addition of $MgSO_4*7H_2O$, $CaCl_2*H_2O$, $Na_2MoO_4*2H_2O$, and $FeSO_4*7H_2O$ at B media concentration to Bg11-N media. "+MgSO4" refers to the addition of $MgSO_4*7H_2O$ at B media concentration to Bg11-N media. "+FeSO4" refers to the addition of $FeSO_4*7H_2O$ at B media concentration to Bg11-

N media. “+Mo” refers to the addition of $Na_2MoO_4*2H_2O$ at B media concentration to Bg11-N media. “-Stock 4” refers to the removal of Citric Acid, $EDTANa_2*2H_2O$, and $FeCl_3$from Bg11-N media.

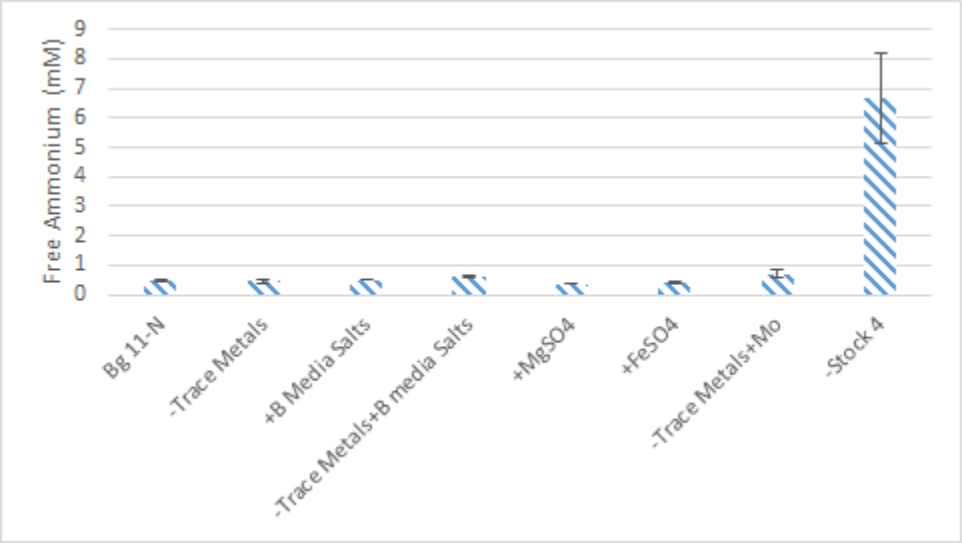

Figure 7: Effect of modified Bg11-N on A. Vinelandii 163 ammonium production

Cultures of A. Vinelandii 163 are grown at 28 °C, 150 rpm, in their respective media conditions, without antibiotics, and with 20 g/L sucrose for 7 days. To ensure strain purity, cultures are taken from B media culture plates that contain kanamycin. Displayed above is the free ammonium concentration at day seven. The ammonium concentration is measured using the reagent assay containing 270 mg of phthalic dicarboxaldehyde, 5 mL of ethanol, 100 mL of 0.2 M phosphate buffer at 7.3 pH, and 50 µL of Beta-mercaptoethanol. “-Trace Metals” refers to the removal of H_3BO_3, $MnCl_2*4H_2O$, $ZnSO_4*7H_2O$, $Na_2MoO_4*2H_2O$, $CuSO_4*5H_2O$, $Co(NO_3)_2*6H_2O$ from Bg11-N media. “+B Media Salts” refers to the addition of $MgSO_4*7H_2O$, $CaCl_2*H_2O$, $Na_2MoO_4*2H_2O$, $FeSO_4*7H_2O$ at B media concentration to Bg11-N media. “+MgSO4” refers to the addition of $MgSO_4*7H_2O$ at B media concentration to Bg11-N media. “+FeSO4” refers to the addition of $FeSO_4*7H_2O$ at B media concentration to Bg11-N media. “+Mo” refers to the addition of $Na_2MoO_4*2H_2O$ at B media concentration to Bg11-N media. “-Stock 4” refers to the removal of Citric Acid, $EDTANa_2*2H_2O$, and $FeCl_3$from Bg11-N media.

As shown in *figure 6* and *figure 7*, the removal of trace metals did not affect the growth rate and ammonium production greatly as the concentration is within the standard deviations of the control, Bg11-N. The addition of the salts from B media shows similar results. However, when the trace metals are removed and the B media salts are added, there is a slight increase in ammonium production to 0.61 mM from the control of 0.47 mM. Similarly, when the trace metals are removed and molybdenum is added back in, the free ammonium production increases from the control condition to 0.69 mM. Of these conditions, the removal of Bg11-N stock 4 showed the greatest increase in free ammonium with an average of 6.67 mM at day seven. Additionally, the removal of stock 4 showed a dramatic decrease in cell density with a final

concentration of 9.57×10^6cells/mL. The removal of stock 4 also resulted in a color change in the media from the common milky yellow to a cloudy green.

4.4 Effect of EDTA, Citric Acid, and Iron (II/III) on Ammonium Production

To further investigate the effect of Stock 4 on the growth and ammonium production of A. Vinelandii 163, we individually added back each component back into the media. Additionally, after removing Stock 4 from Bg11-N media, we decided to add $FeSO_4$, B media's iron source. This additional condition was tested to determine if the alternative oxidation state, Fe(II), would affect growth or ammonium production differently from Fe(III).

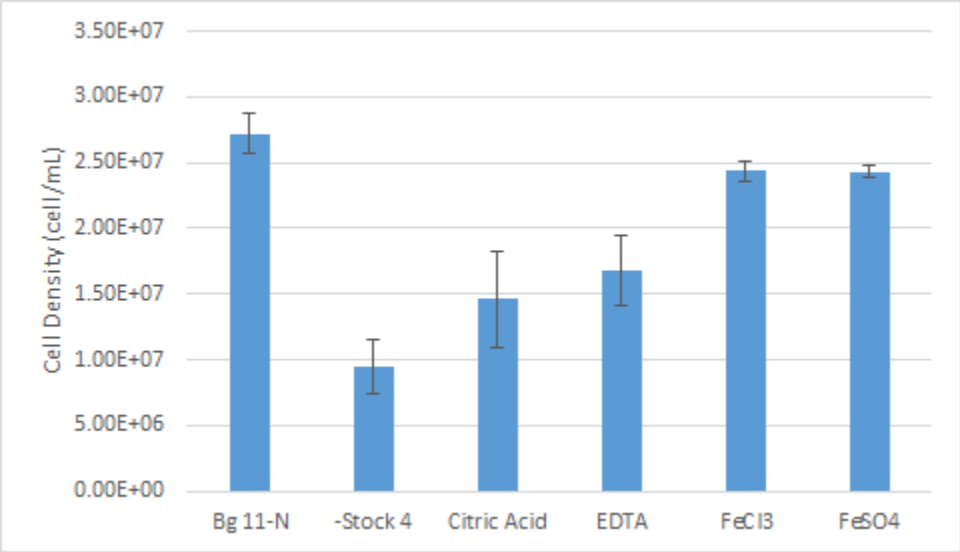

Figure 8:A. Vinelandii 163 growth on Bg11-N-Stock 4 media conditions

Cultures of A. Vinelandii 163 are grown at 28 °C, 150 rpm, in their respective Bg11-N media conditions, without antibiotics, and with 20 g/L sucrose for seven days. To ensure strain purity, cultures are taken from B media culture plates that contain kanamycin. The growth of A. Vinelandii is monitored by measuring the optical density at 600 nm. Displayed above is the cell density at day seven calculated using **Equation 2**. "-Stock 4" refers to the removal of Citric Acid, $EDTANa_2*2H_2O$, and $FeCl_3$from Bg11-N media. Each subsequent condition refers to the removal of Stock 4 and the addition of the stated compound at the standard Bg11-N concentration except for $FeSO_4$, which is added at B media concentrations.

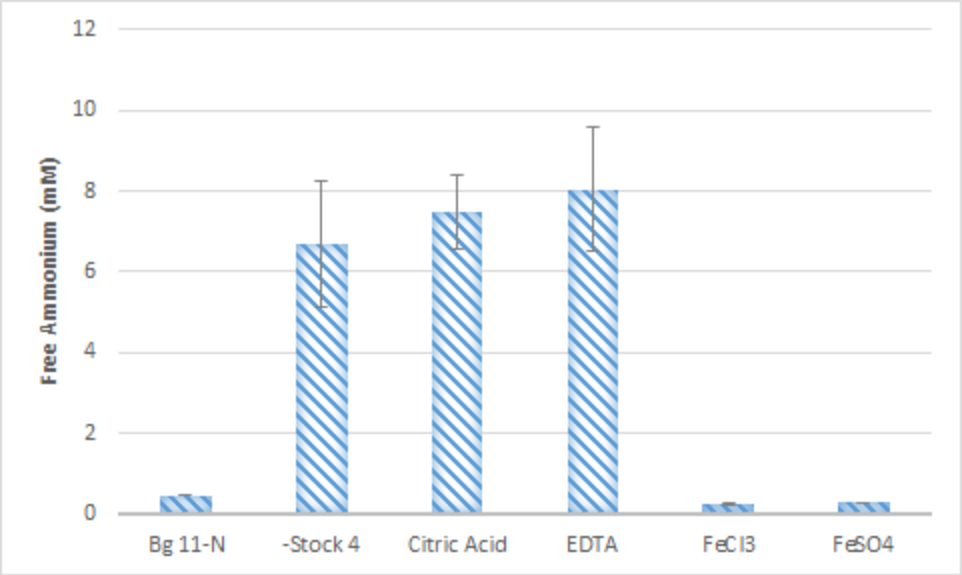

Figure 9:Effect of modified Bg11-N-Stock 4 on A. Vinelandii 163 ammonium production

Cultures of A. Vinelandii 163 are grown at 28 °C, 150 rpm, in their respective media conditions, without antibiotics, and with 20 g/L sucrose for seven days. To ensure strain purity, cultures are taken from B media culture plates that contain kanamycin. Displayed above is the free ammonium concentration at day seven. The ammonium concentration is measured using the reagent assay containing 270 mg of phthalic dicarboxaldehyde, 5 mL of ethanol, 100 mL of 0.2 M phosphate buffer at 7.3 pH, and 50 µL of Beta-mercaptoethanol. "-Stock 4" refers to the removal of Citric Acid, $EDTANa_2*2H_2O$, and $FeCl_3$from Bg11-N media. Each subsequent condition refers to the removal of Stock 4 and the addition of the stated compound at the standard Bg11-N concentration except for $FeSO_4$, which is added at B media concentrations.

As seen in *figure 8*, all conditions had a negative impact on the cell density compared to the control media condition of Bg11-N. In *figure 9*, we see that addition of EDTA and Citric acid both improved the ammonium production in comparison to the "-Stock 4" condition. Interestingly, the addition of $FeCl_3$ and $FeSO_4$ showed cell densities and free ammonium concentration similar to the control condition of Bg11-N at 2.43×10^7cells/mL and 0.26 mM respectively.

4.5 Effect of Molybdenum and Iron Removal on Ammonium Production

As previous results showed that the addition of molybdenum or iron affected the growth and ammonium production, we decided to remove both metals individually from B media. Additionally, we decided to add EDTA to the media to test its effects on Azotobacter's growth and ammonium production.

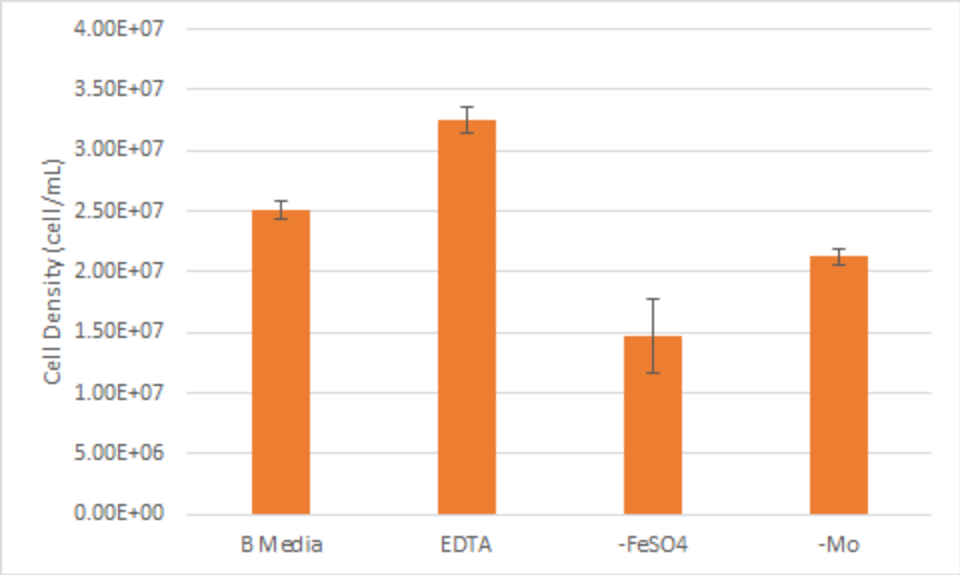

Figure 10:Effect of B media metals on the growth of A. Vinelandii 163

Cultures of A. Vinelandii 163 are grown at 28 °C, 150 rpm, in their respective B media conditions, without antibiotics, and with 20 g/L sucrose for seven days. To ensure strain purity, cultures are taken from B media culture plates that contain kanamycin. The growth of A. Vinelandii is monitored by measuring the optical density at 600 nm. Displayed above is the cell density at day seven calculated using **Equation 2**. "+EDTA" refers to the addition of $EDTANa_2*2H_2O$ at Bg11-N media concentration to B media. "-FeSO4" refers to the removal of $FeSO_4*7H_2O$ from B media concentration. "-Mo" refers to the removal of $Na_2MoO_4*2H_2O$ from B media.

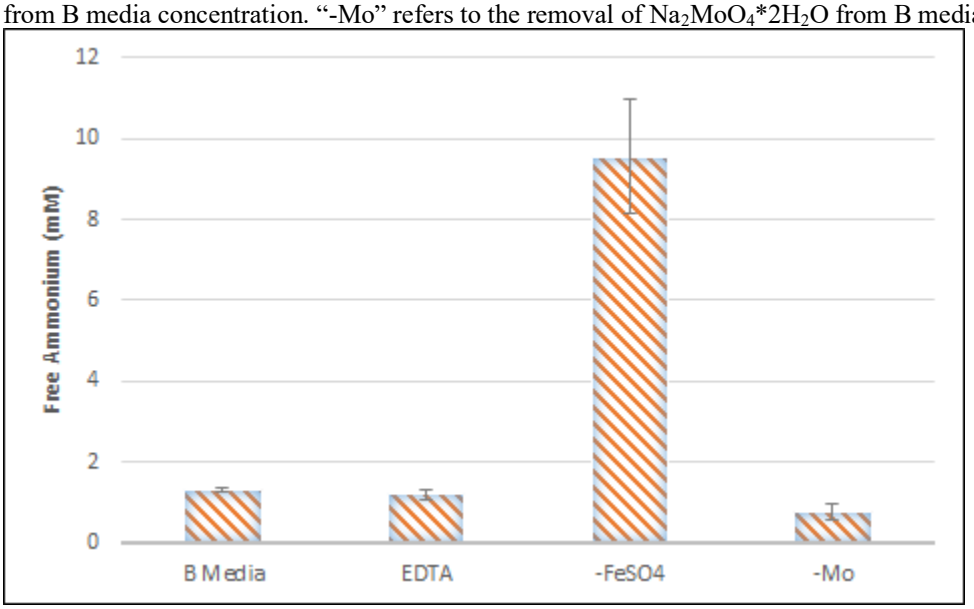

Figure 11:Effect of modified Bg11-N-Stock 4 on A. Vinelandii 163 ammonium production

Cultures of A. Vinelandii 163 are grown at 28 °C, 150 rpm, in their respective media conditions, without antibiotics, and with 20 g/L sucrose for seven days. To ensure strain purity, cultures are taken from B media culture plates that contain kanamycin. Displayed above is the free ammonium concentration at day seven. The ammonium concentration is measured using the reagent assay containing 270 mg of phthalic dicarboxaldehyde, 5 mL of ethanol, 100 mL of 0.2 M phosphate buffer at 7.3 pH, and 50 µL of Beta-mercaptoethanol. "-Stock 4" refers to the removal of Citric Acid, $EDTANa_2*2H_2O$, and $FeCl_3$from Bg11-N media. Each subsequent condition refers to the removal of

Stock 4 and the addition of the stated compound at the standard Bg11-N concentration except for $FeSO_4$, which is added at B media concentrations.

As seen in *figure 10*, the removal of $FeSO_4$ results in a lower cell density of 1.47×10^7cells/mL but greatly improves free ammonium with concentrations of 9.54 mM by day seven. The removal of molybdenum from B media causes a decreased A. Vinelandii 163 cell density at 2.12×10^7cells/mL. However, this cell density is still greater than the cell density in the $FeSO_4$ condition by day seven. Additionally, the removal of molybdenum reduced the free ammonium concentration to 0.757 mM.

Discussion

The ammonium production of Azotobacter Vinelandii is directly dependent on its nitrogen fixing enzyme, nitrogenase. And as discussed before, nitrogenase can be synthesized with three variant forms of dinitrogenase, molybdenum, vanadium, and iron dinitrogenase, which are dependent on the metal present within the media. In the wild type strain of Azotobacter Vinelandii, all three dinitrogenase enzymes are essentially equivalent in their nitrogen fixating ability. However, it is of note that the synthesis of each variant of nitrogenase will inhibit the synthesis of the other two and the synthesis of molybdenum nitrogenase is primarily favored. In our genetically engineered strain, this does not appear to be the case.

In A. Vinelandii 163, the molybdenum nitrogenase expresses a nif+ phenotype, which allows for the continuous production of ammonium without the negative feedback inhibition of nifL. In the case of B media, where there is a source of both molybdenum and iron at concentrations of 0.00022 and 0.005 mM respectively, this would infer that the synthesis of molybdenum nitrogenase would be favored as would the continuous production of ammonium. However, as seen in *figure 11*, we can see that this is not the case. In the B media condition, the free ammonium concentration is one of the lowest compared to the other three conditions. And although, the removal of molybdenum causes an even greater reduction in the free ammonium concentration, we can still infer that the control condition of B media inhibits the nif+ phenotype.

When comparing the free ammonium concentrations of all iron removal conditions, -$FeSO_4$ and -$FeCl_3$, we can assume that the presence of iron is the inhibitory agent. As seen in *figure 9* and *figure 11*, when iron is removed from the media, the free ammonium concentration dramatically increases to 6.67 and 9.54 mM as per the removal of stock 4 and $FeSO_4$ respectively. From these results, we can make the assumption that the higher concentrations of

iron in the media are inhibiting the synthesis of molybdenum nitrogenase and subsequently the nif+ phenotype. Additionally, we can assert that iron nitrogenase are being synthesized in the stead of molybdenum nitrogenase as when A. Vinelandii 163 is grown in the presence of iron, the cell densities are at their highest, as seen in *figures 6*, *8*, and *10*. This higher cell density suggests that A. Vinelandii 163 is growing similarly to its wild type condition, where it will fixate nitrogen sufficient to satisfy its own metabolic needs. However, the higher cell density should not suggest a greater efficiency of nitrogen fixation for iron nitrogenase rather it is most likely a result of the greater concentration of iron in the media.

Conclusion

The purpose of this study was to optimize the ammonium production of Azotobacter Vinelandii 163 and to explore its potential as a nitrogen fixing species in a co-culture setting. To accomplish this goal, we compared two medias, B media and Bg11-N, which when utilized by A. Vinelandii 163 resulted in different concentrations of free ammonium. We then began removing media components from Bg11-N to determine the inhibitory factor, which showed lower concentrations of free ammonium.

From our results, we determined that the iron sources in the media caused a dramatic decrease in ammonium production. This is most likely due to A. Vinelandii utilizing iron to synthesize iron nitrogenase and thereby suppressing the synthesis of molybdenum nitrogenase. Since the synthesis of molybdenum nitrogenase is suppressed, A. Vinelandii 163 cannot express the nif+ phenotype and thusly cannot continuously fix nitrogen.

In order to optimize the ammonium production of Azotobacter Vinelandii 163, the strain must be grown in media devoid of iron. With its current condition, A. Vinelandii 163 cannot be utilized as a nitrogen fixing species in co-culture systems that require iron sources. If A. Vinelandii 163 is to be used as a nitrogen source in co-culture conditions, then the extent of iron's effect on the repressed synthesis of molybdenum nitrogenase would need to be explored.

Future Work

Before experimenting in co-culture conditions, we must evaluate the extent that iron suppresses the synthesis of molybdenum nitrogenase. Our conclusion was that A. Vinelandii 163 utilizes the iron present in the media to synthesize iron nitrogenase over molybdenum nitrogenase, which goes against the established principle that the synthesis of molybdenum nitrogenase is favored over the synthesis of iron nitrogenase. However, some literature suggests that in low concentration of molybdenum, the synthesis of iron nitrogenase can also be favored. To examine this theory further, we should scale the concentrations of molybdenum and iron to determine the point at which the synthesis of molybdenum nitrogenase is favored. With this knowledge, A. Vinelandii 163 could then be utilized as nitrogen source in co-cultures should the new conditions not act adversely to the other co-culture species.

Additionally, when A. Vinelandii 163 is grown without iron sources, this implies that molybdenum nitrogenase is synthesized without iron. However, it is established that the molybdenum nitrogenase contains a MoFe cofactor which acts as the nitrogen fixing anvil in the reduction of nitrogen gas. This could mean that without an iron source, molybdenum nitrogenase synthesizes similarly to iron nitrogenase. The MoFe cofactor in iron nitrogenase does not contain molybdenum and instead has a homologous component. It should be further explored if molybdenum nitrogenase can similarly utilize a homologous element to replace iron in its structure.

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Curriculum vitae

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EDUCATION

Johns Hopkins University, Baltimore, MD
Masters of Science in Engineering, *Chemical and Biomolecular Engineering* Expected Graduation October 2019
Bachelor of Science, *Chemical and Biomolecular Engineering, Minor in Performing/Theater Arts* May 2018

Relevant Course: Transport, Kinetic Processes, Thermodynamics, Modeling & Statistics Analysis, Chemical & Biological Separations, Modeling Dynamics & Control for Chemical and Biological Systems, Metabolic Systems Biotechnology, Cell Biology, Biochemistry, Protein Engineering Lab, Project Design in ASPEN, Product Design

TECHNICAL SKILLS

Software: Matlab, Microsoft Excel, Microsoft Word, SAP Purchasing, MAPLE, ASPEN
Research: Cell Culturing, Anaerobic Digestion Bioreactor (Sampling, Maintenance, and Assembly), Thin Layer Chromatography, qPCR, Drinking Water (Chlorine, BOD, COD, pH), Fluorescent and confocal Microscope Imaging, Microbial Phenotype Identification

RESEARCH AND WORK EXPERIENCE

Betenbaugh Laboratory, Johns Hopkins University Baltimore, MD
Lab Researcher Current Position

- Assessed the viability of algae as a bioremediation solution on the anaerobic digestion of sewage water
- Optimized environment environmental conditions of algae in order to increase biofuel production
- Optimized the ammonium production of Azotobacter and applied its production towards co-culture species for the purposes of increased protein excretion

Johns Hopkins University, Project Design in ASPEN, Projects in ChemE with Experiments Baltimore, MD
Teacher's Assistant 2018 - 2019

- Oversaw and promoted laboratory safety in a project based course consisting of 20 students per class section
- Mentored and assisted undergraduates in bench scale research to solve realistic industry scenarios
- Mentored and assisted undergraduates in modeling and reporting industrial reaction systems of their own design
- Provided technical and linguistic feedback on graded assignments

Griffith Water Treatment Plant Lorton, VA
Engineering Assistant January 2019

- Scripted and tested new Standard Operating Procedures for Granular Activated Carbon Collection and Analysis
- Measured Chlorine Demand to assay the effect of winter temperature on the organic content of the Occoquan River
- Researched and presented alternatives for Sodium Bisulfite as an Ozone quencher in Drinking Water Treatment

National University of Singapore Singapore, Singapore
Lab Researcher Summer 2017

- Confirmed bacterial presence in treated agricultural wastewater to expose health risk towards agricultural workers
- Assessed the viability of the Electro Fenton Reaction as a purification method on anaerobic digested waste
- Mentored and assisted two local High School students in bench scale research and laboratory methods

LEADERSHIP EXPERIENCES

Barnstormers Student Theater Group Baltimore, MD
Executive Board Member, Publicity Manager and Technical Executive 2014-2018

- Oversaw the assignment, documentation, and completion of 5 technical departments projects each with members ranging from 2 to 10 students per each main and offstage performance each semester

MAPP (Mentoring Assistance Peer Program), JUMP-START Baltimore, MD
Mentor, Pre-Orientation Councilor 2015-2017

- Mentored 5 freshman per year both academically and culturally through personal enrichment programs
- Led orientation discussion concerning the balance of student, residential, and Baltimore life at Johns Hopkins

International Thespian Society, Blow by Blow Anti-Bullying Campaign Stafford, VA
Actor, Technical Designer, Group Facilitator 2012

- Led group discussions at local middle schools concerning the effects and signs of bullying and performed the morality play, *Blow by Blow*, of our own creation

Boys Scouts of America Stafford, VA
Eagle Scout 2012

- Drafted, documented, and led the construction of benches for parents and faculty at our local High School's tennis court